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INFLUENCE OF DRUGS ON LIVER-ASCORBIC ACID IN FISH*

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Abstract—The influences of treatment with drugs, of sex, and of treatment with sex hormones on the liver L-ascorbic acid content in fish were studied. An analysis of various enzymatic steps of the glucuronic acid pathway involved in the synthesis of L-ascorbic acid was made. The data obtained, on most points confirmed the findings in this field obtained with mammals. In mammals the drug-induced increase in the activity of drug-metabolizing enzymes in the liver is accompanied by an increased production of L-ascorbic acid. In fish this drug-metabolizing enzyme inducing activity of drugs is only very slight, while there is a clear response in L-ascorbic acid formation. This makes it doubtful whether there is any direct relationship between the induction of an increased activity of drug-metabolizing enzymes in the liver, and the increased formation of L-ascorbic acid in the liver.

A LARGE number of drugs, insecticides and carcinogens are reported to stimulate the activity of drug-metabolizing enzymes in the liver of mammals; this phenomenon has been termed drug induced enhanced drug metabolism.[1–16] Drug treatment also has been shown to be accompanied with increased urinary excretion of L-ascorbic acid in rats, dogs and other animal species[17–27] and increased formation of L-ascorbic acid in rat liver.[21, 23] For the enhanced drug metabolism[23, 24] as well as for the increased L-ascorbic acid excretion[26] observed in mammals after treatment by inducing drugs sex differences occur. These differences disappear in castrated animals but reappear after treatment of these animals with androgens or estrogens.[26, 27]

Liver microsomal drug-metabolizing enzyme activity is extremely low in fish and other aquatic animals[28–30–33] which may be related to the fact that such animals can dispose easily of foreign compounds by excretion through the gills. It is, however, unknown whether these animals have no natural ability to metabolize drugs or whether they have this ability but the ability is not developed under normal conditions. In the latter case it might be expected that long lasting exposure of aquatic animals to drugs or exposure to exceptionally high doses of these drugs will result, by a process of enzyme induction, in a generation of drug-metabolizing enzymes in the liver. Indeed it has been shown[33, 34] that drugs such as DDT, barbiturates, aminopyrine and phenylbutazone, although only weakly, induce the production of *N*-dealkylating, *O*-dealkylating and ring-hydroxylating enzymes in certain fish such as Salmo (trout) and Idus.

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It remains yet to be resolved whether in the fish not only enhanced drug metabolism but also increased formation of L-ascorbic acid occurs after treatment with inducing drugs and whether there is any influence of the sex and of sex hormones on the L-ascorbic acid production. In this relation also the enzyme systems involved in the L-ascorbic acid synthesis, in which the glucuronic acid pathway is involved, are worthwhile examining, since these systems have not yet been examined extensively in fish.

MATERIALS

(a) *Experimental animals*

The types of fish used were *Idus melanotus* (± 200 g) and *Salmo irideus* (± 400 g); they were obtained from the "Koninklijke Nederlandse Heide-Maatschappij", Arnhem, the Netherlands. Adult fishes (± 2yr-old) were used.

(b) *Drugs*

As inducing drugs aminopyrine and barbital were used.

(c) *Hormones*

For treatment of both fish sexes the hormones estradiol benzoate (Dimenformon of Organon) and testosterone propionate (Neohombreol of Organon) in oil were administered.

(d) *Chemicals*

As chemicals were used: uridine-5′-diphosphoglucuronate (UDPGA), uridine-5′-diphosphoglucose (UDPG) and NAD^+, obtained from Boehringer, Mannheim, *D*-glucuronic acid-l-phosphate from California Corporation for Biochemical Research, Los Angeles, U.S.A., phenolphthalein-β-D-glucosiduronate from Serva, Heidelberg, Germany, L-gulono-γ-lactone and adenosine-5′-triphosphate (ATP) from Fluka A.G., Buchs, Switzerland, p-nitrophenol, nicotinamide and D-glucurono-γ-lactone from Sigma Chemical Company, St. Louis, U.S.A., and D-glucuronic acid from British Drug Houses, Poole, England. Sodium gulonate was prepared according to Chatterjee *et al.*[35]

METHODS

(a) The fish were maintained in basins with plain water, into which continuously air was led. The temperature of the water was 17–20°. The drugs were administered orally: barbital in doses of 75 or 150 mg/kg and aminopyrine in doses of 25 or 50 mg/kg. Control animals received water orally. The volume of the solutions administered orally was kept constant for one fish species, namely always 4 ml for *Salmo* and 2 ml for *Idus*. Originally the drugs were applied i.p. In that case the results obtained, however, were extremely variable, which confirms the findings in this respect reported in the literature.[36] Male and female trout were treated with estradiol benzoate 125 μg/kg and testosterone propionate 2·5 mg/kg in oil (injected intramuscularly in the tail muscle) 8 days before the administration of barbital. Control animals received oil applied in the same way. The fish were killed by decapitation 5 hr (in some cases 10 hr) after application of drugs. The liver was quickly excised and weighed, and homogenized with a Potter homogenizer using a Teflon pestle. All the homogenates, except one which will be indicated in the text, were prepared in 0·15 M KCl.

The term homogenate will be used in this sense subsequently in the text. The supernatant fraction obtained by centrifugation for 20 min at 9000 *g* was prepared from the homogenate in a Christ centrifuge. Likewise the term supernatant will be used in this sense subsequently in the text. The supernatant was recentrifuged at 105,000 *g* in a Christ ultracentrifuge with rotor 40 for 60 min, and the pellets obtained were

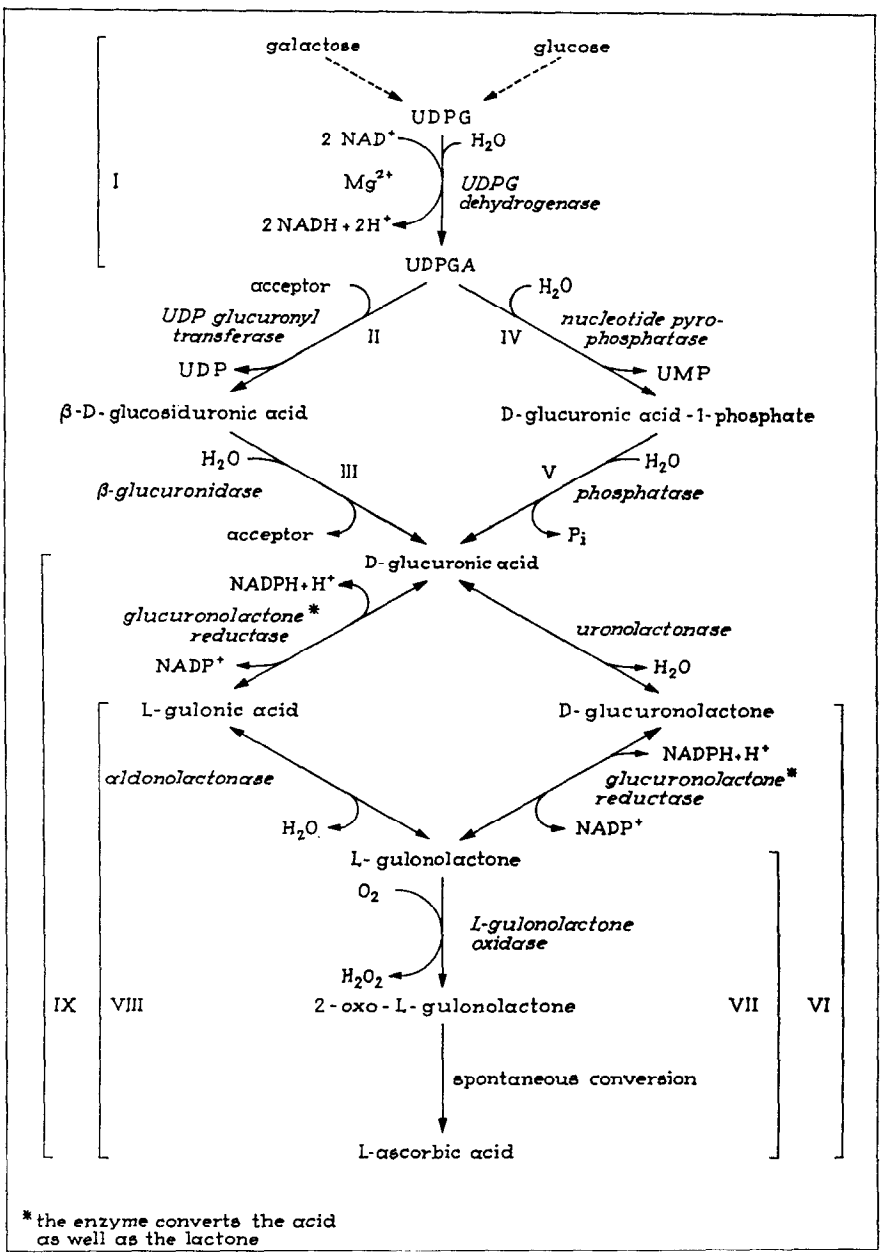

FIG. 1. Schematic representation of the various enzymatic steps involved in the biosynthesis of D-glucuronic acid and L-ascorbic acid reported to occur in mammalian (rat) liver.

resuspended in the suitable medium, 0·15 M KCl as used for homogenate preparation. All preparations were carried out at a temperature of 3–5°.

(b) L-*ascorbic acid determination*

The L-ascorbic acid determination was carried out according to the methods of Roe and Kuether[37] with a modification of Schaffert and Kingsley[38] based upon the reaction between L-ascorbic acid and 2,4-dinitrophenylhydrazine.

TABLE 1. THE INFLUENCE OF DRUG TREAT

Treatment	Oral dose mg/kg	Sex	*n*	L-ascorbic acid liver content (μg/mg wet wt. tissue) *Idus melanotus* 5 hr		*P*
Aminopyrine	0	Male	15	0·1398* ± 0·0084	0·1500† ± 0·0070	—
		Female	14	0·1609 ± 0·0111		
	25	Male	16	0·3329 ± 0·0128	0·3360 ± 0·0120	<0·001
		Female	15	0·3392 ± 0·0202		
	50	Male	16	0·2494 ± 0·0285	0·2510 ± 0·0100	<0·0010
		Female	8	0·2542 ± 0·0180		
Barbital	0	Male	11	0·1270 ± 0·0133	0·1230 ± 0·0120	—
		Female	15	0·1190 ± 0·0243		
	75	Male	16	0·2080 ± 0·0156	0·2093 ± 0·0185	<0·001
		Female	10	0·2113 ± 0·0288		
	150	Male	15	0·2490 ± 0·0202	0·2463 ± 0·0157	<0·001
		Female	6	0·2398 ± 0·0216		

* Mean value ± S.E., calculated for each of the sexes. Applying Student's *t* test no significant differences ($P > 0·05$) were found between male and female.

† Mean value ± S.E., calculated for the total group of fish. Applying Student's *t* test significant differences were found between drug treated and untreated animals. See P values.

n: Number of fish used; P: Calculated by applying Student's *t* test.

(c) *Enzymatic methods*

As indicated in the literature [28,31,39] for the liver enzymes of fish the temperature for their optimal activity is not 37° like in mammals, but 25°. We could confirm this for the steps I, II and III (see Fig. 1), where at a temperature of 37° no difference in activity was found between the control experiments and the experiments in which the substrate was added. Instead of the buffers described in various methods in the literature Tris buffer was used, as advised by Dutton.[39]

The enzymatic conversions:

I. Conversion of UDPG to UDPGA, the step in which the enzyme UDPG dehydrogenase is involved. The method[40] is based on the conversion, by the

supernatant, of NAD^+ to NADH at pH 8·7 and a Mg^{2+} concentration of 0 mM.

II. Glucuronidation of *p*-nitrophenol under formation of a β-D-glucosiduron using UDPGA as D-glucuronic acid donor, the step in which the enzyme U glucuronyltransferase is involved. The method used[40] is based on the measu ment, in the supernatant, of the disappearance of *p*-nitrophenol. The cont contained all the components except *p*-nitrophenol.

THE LIVER ASCORBIC ACID CONTENT OF FISH

				Salmo irideus (trout)		
10 hr		P	*n*	5 hr		P
860* ± 0·0425 1631 ± 0·0630	0·1740† ± 0·0060	—	12 15	0·0930* ± 0·0034 0·1010 ± 0·0099	0·0970† ± 0·0002	
3326 ± 0·0392 3303 ± 0·0190	0·3333 ± 0·0019	<0·001	10 4	0·2400 ± 0·0154 0·2040 ± 0·0225	0·220 ± 0·0130	<0·(
3200 ± 0·0118 2920 ± 0·0109	0·3030 ± 0·0140	<0·001				
0912 ± 0·0088 1029 ± 0·0105	0·0980 ± 0·0060	—	12 15	0·0930 ± 0·0034 0·1010 ± 0·0099	0·097 ± 0·002	
2040 ± 0·0208 2208 ± 0·0161	0·2140 ± 0·0120	<0·001				
2160 ± 0·0184 2151 ± 0·0131	0·2160 ± 0·0080	<0·001	7 5	0·3010 ± 0·0164 0·3290 ± 0·0430	0·3130 ± 0·0030	<0·(

III. Hydrolysis of β-D-glucosiduronic acid under formation of D-glucuronic a in which the enzyme β-glucuronidase is involved. The method used[41] is ba on the measurement of the free phenolphthalein, formed from phenolphthal β-D-glucosiduronate. The substrate concentration used was 0·3 μM. The acti was measured in the homogenate as well as in the pellets.

IV. Conversion of UDPGA to D-glucuronic acid-1-phosphate, a step in wh a nucleotide pyrophosphatase is involved. The method used[40] is based on measurement, in the supernatant, of the inorganic phosphate (P_i) form which was determined according to Fiske and SubbaRow.[42]

V. Conversion of D-glucuronic acid-l-phosphate to D-glucuronic acid, a step

which a phosphatase is involved. Enzymatic hydrolysis of D-glucuronic acid-l-phosphate by the supernatant was measured according to the method described under IV.

VI. Formation of L-ascorbic acid from D-glucuronolactone.

VII. Formation of L-ascorbic acid from L-gulonolactone.

VIII. Formation of L-ascorbic acid from L-gulonic acid.

IX. Formation of L-ascorbic acid from D-glucuronic acid.

The method used in VI–IX is based on the measurement of L-ascorbic acid, as mentioned before and under experimental conditions as described by Stirpe and Comporti. The substrates used were D-glucuronolactone, L-gulonolactone, L-gulonic acid and D-glucuronic acid respectively. The conversions indicated as VI, VII, VIII and IX were measured in suspended pellets obtained from sucrose-Tris buffer homogenate. Moreover, although indicated as lesss uitable for this purpose,[43] the conversions VI and VII were measured in suspended pellets obtained from KCl homogenate, too. With both procedures no activity was found for step VII

RESULTS

The results summarized in Table 1 show that after aminopyrine and barbital in the doses mentioned, a significant increase in the L-ascorbic acid content of the liver takes place. A comparison of the liver L-ascorbic acid contents found for untreated male and female fish with those described in the literature for male and female rat,[23, 25] gives for male and female fish $\pm$ 40% and $\pm$ 60% of the rat liver content respectively. From the experimental data concerning male and female fish, it may be concluded that no sex differences are detectable in the L-ascorbic acid liver content, neither for the untreated fish, nor for the fish treated with aminopyrine and barbital.

Since in rat increased formation of L-ascorbic acid in the liver under influence of the drugs mentioned is reported to be associated with increased excretion of L-ascorbic acid in the urine, we liked to examine whether the same holds true for fish. We did not succeed however, in measuring the L-ascorbic acid excretion in the urine of fish because of technical difficulties. Therefore experiments were done in which 5 fishes (*Idus melanotus*), total weight $\pm$ 1000 g, were confined to a volume of 400 ml water. Treatment of these fishes with aminopyrine and barbital did not result in a detectable excretion of L-ascorbic acid in the surrounding water. The influence of estradiol and testosterone in barbiturate treated trouts on the L-ascorbic acid content in the liver is given in Table 2. Both testosterone and estradiol decrease significantly the liver content of the acid.

The formation of L-ascorbic acid in mammals is tied up intricately to the glucuronic acid pathway in which UDPG is converted by a sequence of enzymatic steps to D-glucuronic acid which in its turn is converted to L-gulonic acid which probably via L-gulonolactone leads to L-ascorbic acid (see Fig. 1).[43–46] The question arises whether in the fish, studied by us, the same type of enzymes and the same intermediate products are involved. The enzymes studied were those involved in the metabolic conversions in the steps indicated in Fig. 1 with I–IX. The results are summarized in Table 3. This table shows that all steps could be detected with the exception of step VII and VIII. Step VII could not be detected neither in pellets obtained from KCl homogenate nor in pellets obtained from sucrose-Tris buffer homogenate. Step VIII

was only studied in pellets from sucrose-Tris buffer homogenate. Also the influence of pretreatment (5 hr) with aminopyrine and barbital applied orally, was studied for several steps (I, III, VI and VII).

The results, which also are summarized in Table 3, show that after barbiturate treatment there is an increased conversion in the steps I, III and VI and after aminopyrine in the steps I and VI, while for step VII neither in the livers of aminopyrine

TABLE 2. THE INFLUENCE OF PRETREATMENT WITH TESTOSTERONE OR ESTRADIOL ON THE BARBITAL-INDUCED INCREASE IN THE LIVER ASCORBIC ACID CONTENT OF FISH (*Salmo irideus*, TROUT)

Treatment	dose mg/kg	*n*	L-ascorbic acid liver content μg/mg wet wt. tissue	P
Barbital	150	12	0·3130 ± 0·0030	—
Testosterone + Barbital	2·5 150	6	0·1384 ± 0·0120	< 0·001
Estradiol + Barbital	0·125 150	5	0·1064 ± 0·0010	< 0·001

n: number of trout used; P Calculated by applying Student's *t* test.
The values given are means ± S.E.

pretreated nor in the livers of barbiturate pretreated animals, any conversion could be detected.

DISCUSSION

It has been shown that oral administration of aminopyrine (25 and 50 mg/kg) and barbital (75 and 150 mg/kg) to *Idus melanotus* and *Salmo irideus* (trout) doubled the ascorbic acid content in the liver of these fishes within 5–10 hr; similar observations have been described for the rat. It is accepted that the enhanced L-ascorbic acid content in rat liver caused by drug treatment is the consequence of an enhanced biosynthesis[23, 24, 27] which is probably the direct consequence of an enhanced formation of D-glucuronic acid in the liver.[24, 27] The finding that in fish liver, with the exception of step VII and step VIII, which could not be detected in fish, the same enzyme systems (see Fig. 1) are involved in the L-ascorbic acid synthesis as in rat liver indicates that probably the ascorbic acid synthesis in fish and mammals takes place along practically identical pathways. Whether there are real differences between fish and mammals for the steps VII and VIII or whether the methods applied are less suitable for fish liver homogenates, requires further investigation.

After treatment (5 hr) of the fishes with aminopyrine a significant increase in the activity of the steps I and VII could be detected. After barbital treatment similarly an increase in the activity of the steps I, III and VI was detected. In the rat after drug treatment an increased activity is reported in step I, however, only after chronic (at least some days) treatment.[17, 24, 27, 47, 48] The conversion of L-gulonolactone to L-ascorbic acid, step VII—which could not, as mentioned, be detected in the untreated fish—also was not detectable 5 hr after treatment with aminopyrine or barbital.

A sex difference in the L-ascorbic acid content of the liver of untreated and drug-treated mammals as reported in literature[23–25] was found neither in the untreated nor in the drug-treated fish.

TABLE 3. THE VARIOUS STEPS INVOLVED IN THE ASCORBIC ACID BIOSYNTHESIS DETECTED IN FISH LIVER (*Salmo irideus*, TROUT)

Step	Enzymatic conversion	Fraction used (Prepared with KCl)	Expression activity	Treatment: None		Aminopyrine 25 mg/kg orally		Barbital 150 mg/kg orally	
				n	Activity	*n*	Activity	*n*	Activity
I	UDPG dehydrogenase	Supernatant	μM UDPG/min/g wet wt. tissue	10	0·0184 ± 0·0014	5	0·0227 ± 0·0014 (0·05 > P > 0·02)	5	0·0269 ± 0·0019 (0·01 > P > 0·001)
II	UDP glucuronyl transferase	Supernatant	μM *p*-NP/min/g wet wt. tissue	5	0·2590 ± 0·0261				
III	β-Glucuronidase	Homogenate	μg phenolphthalein/min/g wet wt. tissue	7	0·0214 ± 0·0010	5	0·0245 ± 0·0017 (0·2 > P > 0·1)	5	0·0254 ± 0·0002 (0·01 > P > 0·001)
		Pellets		4	0·0064 ± 0·0006	5	0·0094 ± 0·0019	5	0·0099 ± 0·0011
IV	Nucleotide pyrophosphatase	Supernatant	μM phosphate/min/g wet wt. tissue	15	0·1525 ± 0·0099		(0·2 > P > 0·1)		(0·05 > P > 0·02)
V	Phosphatase	Supernatant	μM phosphate/min/g wet wt. tissue	6	0·1065 ± 0·0053				
VI	From D-glucurono-lactone to L-ascorbic acid	Pellets	μg ascorbic acid/min/mg wet wt. tissue	27	0·0038 ± 0·0002	14	0·0063 ± 0·0003 (P < 0·001)	12	0·0054 ± 0·0002 (P < 0·001)
VII	From L-gulono-lactone to L-ascorbic acid	Pellets	μg ascorbic acid/min/mg wet wt. tissue	27	No activity	14	No activity	12	No activity
		(Prepared with sucrose-Tris buffer)							
VI	From D-glucurono-lactone to L-ascorbic acid	Pellets	μg ascorbic acid/min/mg wet wt. tissue	17	0·0063 ± 0·0008				
VII	From L-gulono-lactone to L-ascorbic acid	Pellets	μg ascorbic acid/min/mg wet wt. tissue	17	No activity				
VIII	From L-gulonic acid to L-ascorbic acid	Pellets	μg ascorbic acid/min/mg wet wt. tissue	16	No activity				
IX	From D-glucuronic acid to L-ascorbic acid	Pellets	μg ascorbic acid/min/mg wet wt. tissue	16	0·0074 ± 0·0006				

The enzymatic activities given as rates of conversion, were calculated by a graphic method and expressed as the initial velocity.
The values given are means ± S.E.
n: Number of trout used; P: Calculated applying Student's *t* test. Groups of fish treated with aminopyrine and barbital were compared with the group of untreated fish.

The drugs used are reported to increase drug-metabolizing enzyme activity in the liver of mammals, an activity which appears to be accompanied with increased formation and excretion of L-ascorbic acid. In fish the drug-metabolizing enzyme inducing activity of drugs, although detectable, is weak.[33] Drug influence on the L-ascorbic acid content in fish liver, however, is very clear. This makes it doubtful whether there is any direct relationship between the induction by drugs of an increased activity of drug-metabolizing enzymes in the liver and the increased formation of L-ascorbic acid in the liver after drug treatment.

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